Supporting Information

Identification of Tetrahydropyrido[4,3-*d*]pyrimidine Amides as a New Class of Orally Bioavailable TGR5 Agonists

David W. Piotrowski,* Kentaro Futatsugi, Joseph S. Warmus, Suvi T. M. Orr, Kevin D. Freeman-Cook, Allyn T. Londregan, Liuqing Wei, Sandra M. Jennings, Michael Herr, Steven B. Coffey, Wenhua Jiao, Gregory Storer, David Hepworth, Jian Wang, Sophie Y. Lavergne, Janice E. Chin, John R. Hadcock, Martin B. Brenner, Angela C. Wolford, Ann M. Janssen, Nicole S. Roush, Joanne Buxton, Terri Hinchey, Amit S. Kalgutkar, Raman Sharma, Declan A. Flynn

Pfizer Worldwide Research and Development, Groton, CT 06340

Table of Contents:

General Methods:

All chemicals, reagents and solvents were purchased from commercial sources and used without further purification. All reactions were performed under an atmosphere of nitrogen unless otherwise noted. Nuclear magnetic resonance spectra (^{1}H, ^{19}F, ^{13}C NMR) were recorded with 400 MHz and 500 MHz Varian or Bruker spectrometers. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. ^{19}F NMR spectrum is referenced against the solvent deuterium signal, with $CFCl_3$ set

to 0 ppm. The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet. Due to differences in solvents used in sample preparation and, in some cases amount of water present, not all exchangeable protons were observable. Mass spectrometry (MS) was performed via atmospheric pressure chemical ionization (APCI) or electron scatter (ESI) ionization sources. Liquid chromatography mass spectrometry (LCMS) was performed on an Agilent 1100 Series (Waters Atlantis C18 column, 4.6 x 50 mm, 5 μm; 95% water/acetonitrile linear gradient to 5% water/acetonitrile over 4 min, hold at 5% water/ 95% acetonitrile to 5.0 min, trifluoroacetic acid modifier (0.05%); flow rate of 2.0 mL/ min). Silica gel chromatography was performed using a medium pressure Biotage or ISCO system using columns pre-packaged by various commercial vendors including Biotage and ISCO. Whatman pre-coated silica gel plates (250 μm) were used for analytical TLC.

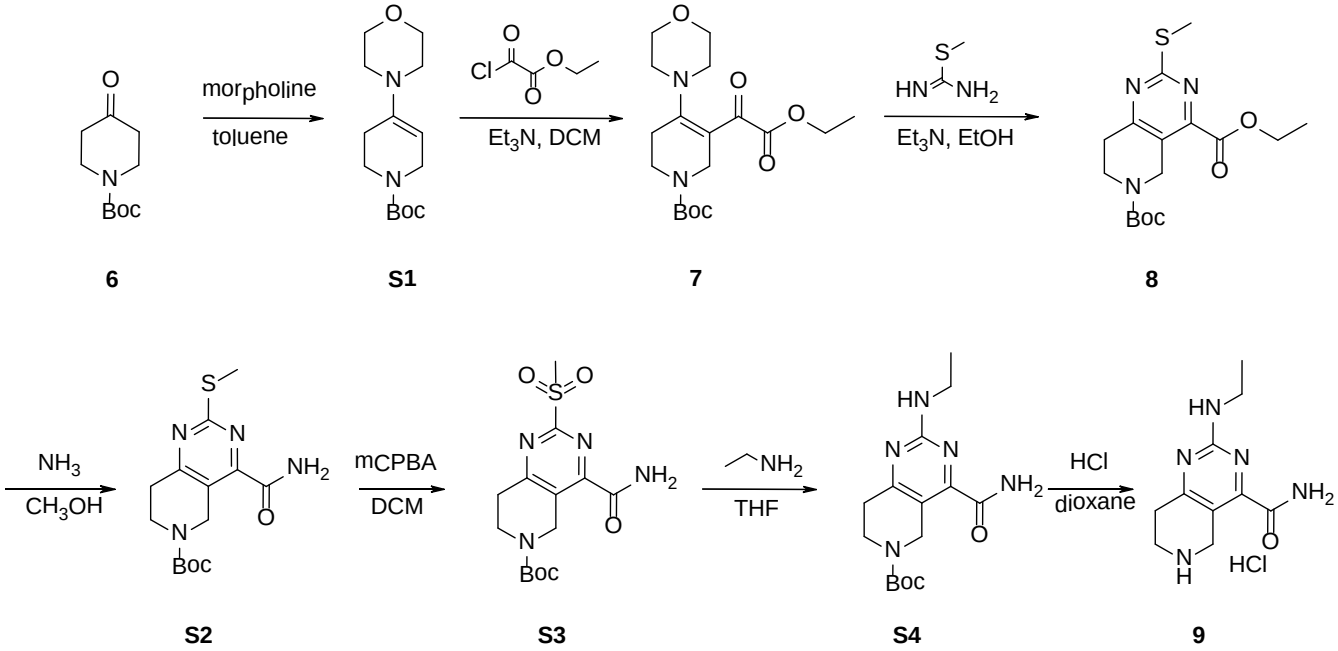
All library compounds were > 85% purity as judged by LCMS. Most of the library compounds, except for **13** and **15**, were re-prepared by the experimental methods described below.

The ^{1}H NMR and MS data for compounds **1-3** were consistent with data reported in the literature.[1]

For human blood donations, documented informed consent of the donor was obtained in accordance with recognized international standards for the protection of human research subject(s).

Section 1: Synthesis of Compounds and Intermediates

Synthesis of Intermediate 9



To a solution of Boc-piperidin-4-one **6** (500 g, 2.51 mol) in toluene (2 L) was added morpholine (250 g, 2.76 mol) and the flask was equipped with a Dean-Stark trap and the mixture was stirred under reflux for 18 h. The mixture was cooled to room temperature, and the solvent was removed under reduced pressure to give enamine **S1** (720 g) as a yellow liquid. This material was used without further purification.

To a solution of enamine **S1** (720 g) and triethylamine (327 g, 3.23 mol) in dichloromethane (2 L) was added ethyl oxalyl chloride (405 g, 2.96 mol) dropwise at 0 °C, and the mixture was allowed to warm to room temperature while stirring for 18 h. The mixture was filtered and the filtrate was concentrated under reduced pressure to give compound **7** (250 g) as a yellow solid. This material was used without further purification.

To a solution of compound **7** (1100 g, 3 mol) in ethanol (4 L) was added S-methylthiourea (562 g, 3 mol) and triethylamine (1200 g, 11.9 mol) and the mixture stirred under reflux for 18 h. The mixture was cooled to room temperature and filtered to give a yellow solid that was washed with

dichloromethane (2 L). The filtrate was concentrated under reduced pressure to give compound **8** (250 g) as a yellow solid. This material was used without further purification.

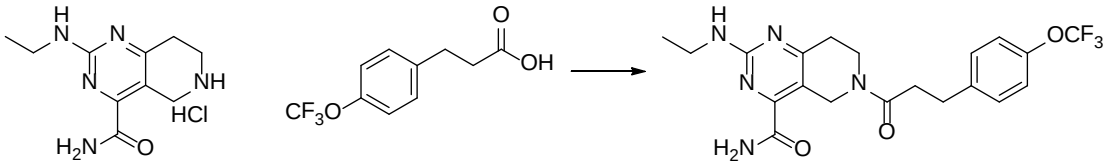
To a solution of compound **8** (120 g, 340 mmol) in methanol (3 L) was added a solution of 7 N ammonia in methanol (500 mL, 3500 mmol), and the mixture was stirred in a sealed bottle at 40 °C for 18 h. The mixture was cooled to room temperature and the solvent was removed under reduced pressure to give compound **S2** (110 g) as a white solid. This material was used without further purification.

To a solution of compound **S2** (108 g, 333 mmol) in dichloromethane (2 L) was added *m*-chloroperbenzoic acid (145 g, 767 mmol) in portions at 0 °C, and the mixture was allowed to warm to room temperature while stirring for 2 h. The reaction mixture was poured into a separatory funnel and washed with sat. aq. $Na_2S_2O_3$ (2 x 300 ml), sat. aq. K_2CO_3 (2 x 300 mL), dried (Na_2SO_4) and concentrated under reduced pressure to give compound **S3** (118 g) as a white solid. This material was used without further purification.

To a solution of compound **S3** (40 g, 112 mmol) in THF (1.5 L) was added ethylamine (68 g, 1500 mol), and the mixture was stirred at 80 °C for 18 h. The solvent was removed under reduced pressure and the residue was crystallized from THF and ethyl acetate to give compound **S4** (22 g, 61%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (br s, 1H), 5.56 (br s, 1H), 5.06 (br s, 1H), 4.95-4.91 (m, 2H), 3.72-3.67 (m, 2H), 3.47-3.40 (m, 2H), 2.84-2.82 (m, 2H), 1.48 (s, 9H), 1.27-1.19 (m, 3H).

To a solution of compound **S4** (140 g, 436 mmol) in dichloromethane (400 mL) was added 4.0 M HCl in dioxane (1.5 L) dropwise at 0 °C, and the mixture was stirred at room temperature for 6 h. The mixture was filtered to give a solid that was further dried in a vacuum oven to give compound **9** (122 g, 96%) as a white solid. ^{1}H NMR (D_2O, 400 MHz) δ 4.47 (s, 2H), 3.47 (t, J = 6.8 Hz, 2H), 3.35 (q, J = 7.2 Hz, 2H), 3.05 (t, J = 6.8 Hz, 2H), 1.09 (t, J = 7.2 Hz, 3H); m/z = 222.1 $(M+H)^+$.

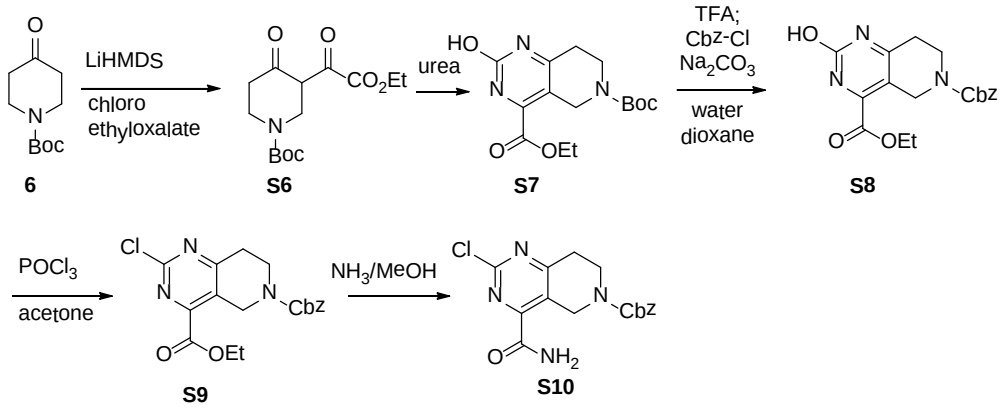
Synthesis of Compound 16



9 **S5** **16**

To a solution of the amine **9**-HCl salt (10.0 g, 34 mmol) in dichloromethane (250 mL) were added 4-trifluoromethoxyhydrocinnamic acid **S5** (8.9 g, 38 mmol), triethylamine (12 mL, 86 mmol), DMAP (5.2 g, 41 mmol), and EDCI (9.7 g, 48 mmol). The resulting mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with ethyl acetate (300 mL) and 1 M aqueous HCl solution (300 mL) was added. The layers were separated and the organic layer was washed with 1N NaOH (300 mL), brine (300 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a clear oil. The crude material was purified by ISCO Combiflash system eluting with heptanes/acetone gradient to give 13.2 g of compound **16** as clear oil that solidified upon standing. This material was recrystallized from acetonitrile to give 8.25 g (56%) of **16** as a white solid. mp 132-133 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.70 (br s, 1H), 7.18 (d, *J* = 8 Hz, 2H), 6.98 (d, *J* = 8 Hz, 2H), 4.68 (br s, 1H), 4.11 (q, *J* = 7.0 Hz, 2H), 3.46-3.42 (m, 1H), 3.10-2.96 (m, 2H), 2.76-2.69 (m, 1H), 2.03-1.97 (m, 3H), 1.65-1.62 (m, 2H), 1.24 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 170.9, 168.1, 167.2, 159.7, 150.7, 147.5, 139.7, 129.8, 120.8, 120.4 (q, $^1J_{CF}$ = 257 Hz), 115.6, 44.7, 38.5, 36.3, 35.0, 32.2, 30.9, 14.8; ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ -57.9; HPLC (purity) 99.3%; HRMS Calcd. for $C_{20}H_{22}F_3N_5O_3$ $(M+H)^+$ 438.1748; Found 438.1734; Anal. Calcd. for $C_{20}H_{22}F_3N_5O_3$: C; 54.92, H; 5.07, N; 16.01. Found, C; 54.91, H; 5.01, N; 16.00.

Synthesis of Intermediate S10



To a solution of N-Boc-piperid-4-one **6** (1.5 kg, 7.5 mol) in THF (1 L) was added LiHMDS (7.5 L, 7.5 mol) dropwise at -78 °C under N_2 atmosphere, and the mixture was stirred for 1 h. Ethyl chlorooxalate (1.1 kg, 7.5 mol) was added at -78 °C, and stirring was continued for 5 h while allowing the cooling bath to warm to room temperature. Water (1 L) was added and the mixture was extracted with MTBE (2 x 1 L). The combined organic layers were washed (brine), dried (Na_2SO_4) and concentrated under reduced pressure to give compound **S6** (1.5 kg, 67%) as a yellow oil. m/z = 298.1 $(M-H)^-$.

To a solution of compound **S6** (500 g, 1.67 mol) in AcOH (1 L) was added urea (150 g, 2.51 mol) and the mixture was stirred at 50 ^oC for 18 h. The solvent was removed under reduced pressure and the residue was dissolved in dichloromethane (500 mL). The organic layer was washed with water (500 mL), dried (Na_2SO_4) and concentrated to give a residue that was purified via silica gel chromatography eluting with dichloromethane/methanol (20:1) to give **S7** (150 g) as a black solid.

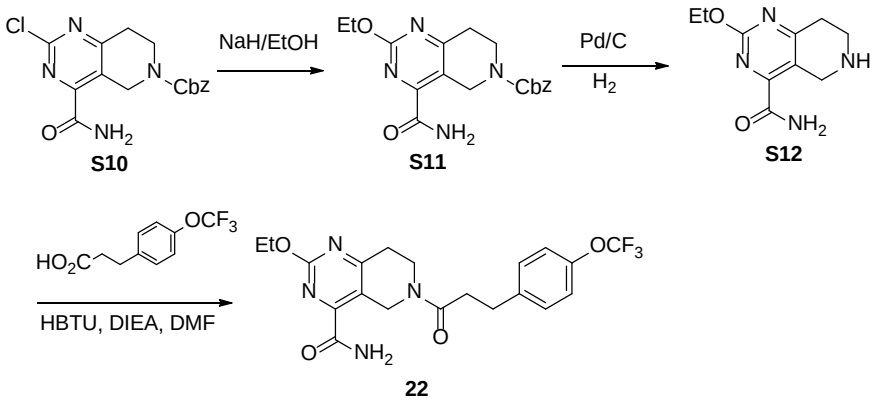
To a solution of compound **S7** (156 g, 0.48 mol) in dichloromethane (1.5 L) was added TFA (550 g, 4.8 mol) at room temperature, and the mixture was stirred for 18 h. The solvent was removed under reduced pressure to give 167 g of a residue that was used in the subsequent step without further purification. This material was dissolved in water (500 mL) and dioxane (500 mL). Benzyl chloroformate (127 g, 0.74 mol) and Na_2CO_3 (106 g, 1.0 mol) were added and the mixture was stirred at room temperature for 18 h. Water (300 mL) was added and the mixture was extracted with

dichloromethane (2 x 500 mL). The combined organic layers were washed (brine), dried (Na_2SO_4) and concentrated to give a residue that was purified via silica gel column chromatography eluting with dichloromethane/methanol (20:1) to give **S8** as a black solid.

To a solution of **S8** (45 g, 126 mmol) in acetonitrile (500 mL) was added $POCl_3$ (45 mL, 491 mmol) and the mixture was heated under reflux for 2 h. The solvent was removed under reduced pressure and the residue was purified via silica gel column chromatography, eluting with petroleum ether/ethyl acetate (3:1) to give **S9** (22.7 g, 50%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.31 (m, 5H), 5.18 (s, 2H), 4.96 (s, 2H), 4.46 (q, *J* = 7.2 Hz, 2H), 3.84 (t, *J* = 6.0 Hz, 2H), 3.06 (br s, 2H), 1.41 (t, *J* = 7.2 Hz, 3H); m/z = 376.0 $(M+H)^+$.

A mixture of **S9** (2.15 g, 5.72 mmol) and 7 N ammonia in MeOH (8.5 mL, 60.2 mmol) was heated in a microwave at 50 C for 2 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure to give **S10** as a beige solid (2.00 g, >99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (br s, 1H), 7.38-7.30 (m, 5H), 5.59 (br s, 1H), 5.20-5.17 (m, 4H), 3.82 (t, *J* = 8.0 Hz, 2H), 3.12-3.03 (m, 2H). m/z = 347.2 $(M+H)^+$.

Synthesis of 22



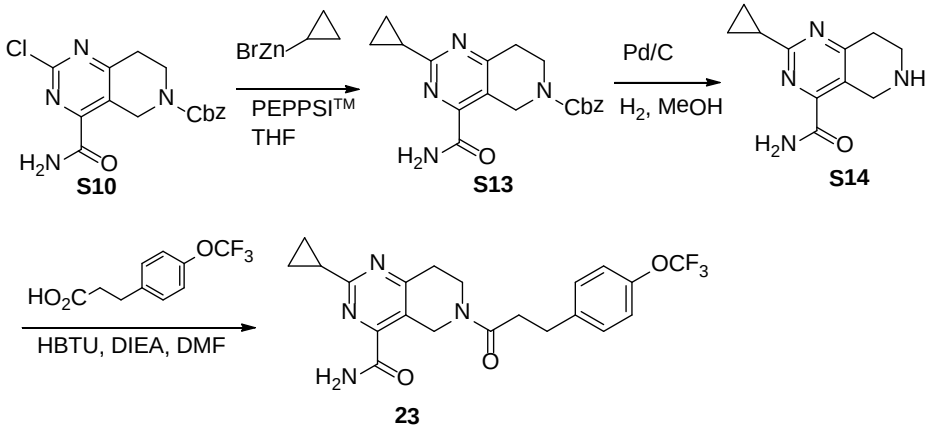
Intermediate **S10** (1.00 g, 2.88 mmol) was added to a mixture of sodium hydride (60% in mineral oil, 461 mg, 11.5 mmol) in ethanol (15 mL) at room temperature, and the mixture was stirred at room temperature for 18 h. HCl (5 mL, 4 M in dioxane) was added, and the mixture was concentrated under reduced pressure to give a yellow solid. The residue was dissolved in ethyl acetate (50 mL) and water

(50 mL), and the aqueous layer was extracted with ethyl acetate (3 x 25 mL). The combined organic layers were concentrated to dryness to give **S11** (1014 mg, 98%) as a golden viscous oil that was used without further purification. m/z = 357.2 $(M+H)^+$.

To a solution of **S11** (1.00 g, 2.81 mmol) in ethanol (100 mL) was added 200 mg of Pd/C (50% wet for safety) in a Parr bottle, and the mixture was shaken under hydrogen atmosphere (45 PSI) at room temperature for 18 h. The mixture was filtered through a pad of celite and the solvent was removed under reduced pressure to give **S12** as a brown solid (615 mg, 98%) that was used without further purification. m/z = 223.2 $(M+H)^+$.

To a mixture of **S12** (263 mg, 1.18 mmol) and 4-trifluoromethoxyhydrocinnamic acid (281 mg, 1.20 mmol) in DMF (10 mL) was added triethylamine (0.50 mL, 3.60 mmol) and HBTU (397 mg, 1.24 mmol) and the reaction mixture was stirred at room temperature for 18 h. The mixture was concentrated under reduced pressure and the residue was purified by ISCO Combiflash system eluting with a gradient of 90-100% ethyl acetate in heptanes to give 158 mg (31%) of **22** as a white solid. HPLC (purity) 98%. ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.13-8.11 (m, 1H), 7.80-7.77 (m, 1H), 7.35 (d, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.12 (d, *J* = 8.0 Hz, 1H), 4.84 (s, 2H), 4.36 (q, *J* = 4.0 Hz, 2H), 3.72 (t, *J* = 6.0 Hz, 2 H), 2.85-2.80 (m, 3H), 2.76-2.73 (m, 2H), 2.67-2.64 (m, 1H), 1.29 (t, *J* = 4.0 Hz, 3 H). m/z = 439.2 $(M+H)^+$.

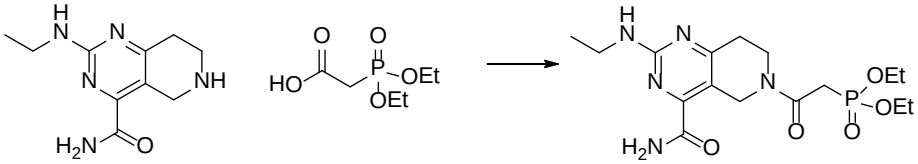
Synthesis of 23



A mixture of **S10** (347 mg, 1.00 mmol) PEPPSI™-IPr catalyst (13.6 mg, 0.02 mmol) in THF (6 mL) was purged with a stream of N_2 and evacuated three times, and stirred at room temperature for 30 min. Cyclopropylzinc bromide (6.0 mL, 3.00 mmol, 0.5 M in THF) was added and the mixture was purged with a stream of N_2 and evacuated three times, then stirred at 65 C for 18 h. After cooling down to room temperature, the reaction was quenched with saturated aqueous NH_4Cl (20 mL), and extracted with ethyl acetate (2 x 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated to give a crude product, that was purified by ISCO Combiflash system eluting with 0-100% ethyl acetate in heptanes to give 127 mg (40%) of **S13** as a white solid. m/z = 353.2 $(M+H)^+$.

S13 was treated in analogous manner to **S11** (hydrogenation and amide coupling) to give 136 mg (77%) of **23** as a white solid. ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.16-8.13 (m, 1H), 7.81-7.79 (m, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 8.0 Hz, 1H), 4.92-4.90 (m, 2H), 3.75 (t, *J* = 6.0 Hz, 2 H), 2.89-2.80 (m, 3H), 2.79-2.75 (m, 2H), 2.71-2.68 (m, 1H), 2.20-2.15 (m, 1H), 1.07-1.01 (m, 4 H). m/z = 435.1 $(M+H)^+$. LCMS = > 95% purity.

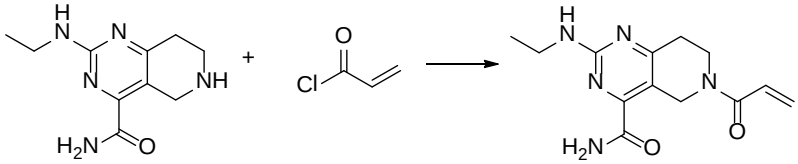
Synthesis of Library Intermediate 10



A mixture of the amine **9** (4.00 g, 13.8 mmol) in DMF (30 mL) at 0 ^{o}C was treated with *i*PrEt$_2$N (5.34 g, 41.4 mmol) and BOP (6.40 g, 15.2 mmol) followed by 2-(diethoxyphosphoryl)acetic acid (2.70 g, 13.8 mmol). The reaction was warmed to room temperature and stirred overnight. The reaction was treated with saturated sodium bicarbonate (50 mL) and extracted with DCM:MeOH 6:1 (5 x 200 mL). The combined organics were washed with brine (100 mL), dried (Na_2SO_4) and evacuated. The crude residue was triturated with a mixture of EtOAc:MeOH (5:1, 6 mL) and vacuum filtered to afford **10** as a white solid (3.55 g, 96%).

^{1}H NMR (400 MHz, d_6-DMSO) δ 7.91 (br s, 1H), 7.68 (br s, 1H), 7.13 (br s, 1H), 4.86 (s, 1H), 4.75 (s, 1H), 4.08 - 3.89 (m, 4H), 3.77 (t, *J* = 5.9 Hz, 1H), 3.72 (t, *J* = 6.1 Hz, 1H), 3.35-3.30 (m, 4H), 2.83 (t, *J* = 5.7 Hz, 1H), 2.70 - 2.64 (t, *J* = 6.3 Hz, 1H), 1.24 - 1.19 (t, *J* = 6.3 Hz, 3H), 1.17-1.14 (t, *J* = 6.8 Hz, 3H), 1.11-1.08 (t, *J* = 7.0 Hz, 3H). m/z = 400.2 $(M+H)^+$. HPLC purity > 95%.

Synthesis of Library Intermediate 11

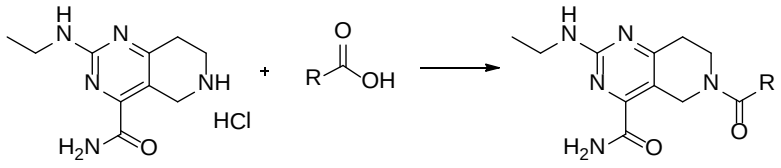


9 **11**

A mixture of the amine **9** (5.00 g, 22.6 mmol) in acetone (80 mL) was treated with saturated sodium carbonate solution (35 mL) followed by acryloyl chloride (3.18 mL, 39.6 mmol). The mixture was stirred at room temperature for 5 hours and then filtered through a Celite pad. After rinsing with acetone (20 mL), the filtrate was concentrated to remove the organic solvent, resulting in the precipitation of light-yellow solids. These solids were collected by vacuum filtration, air-dried, and then recrystallized from EtOAc (20 mL) to afford **11** as a white solid (4.00 g, 64%).

^{1}H NMR (400MHz, CD_3OD) δ 6.88-6.73 (m, 1H), 6.21 (dd, *J* = 17.2, 4.7 Hz, 1H), 5.75 (d, *J* = 10.7 Hz, 1H), 5.05 (d, *J* = 22.2 Hz, 2H), 3.89 (t, *J* = 5.6 Hz, 2H), 3.41 (q, *J* = 7.1 Hz, 2H), 2.90-2.80 (m, 2H), 1.19 (t, *J* = 7.2 Hz, 3H). m/z = 276.3 $(M+H)^+$. LCMS > 95% purity.

HATU coupling Library Protocol



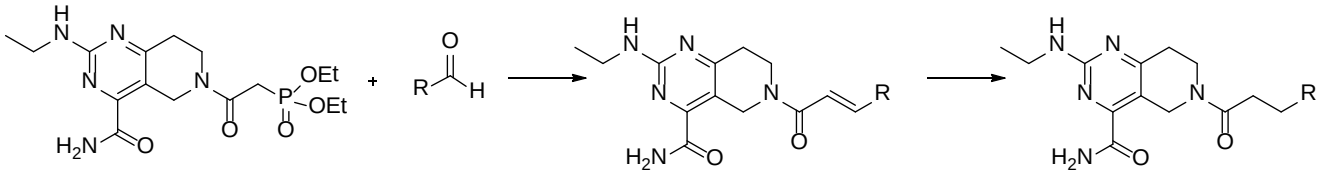
9

To an 8 mL vial containing a DMA solution of the carboxylic acid (600 µL, 90 µmol, 1.2 equiv) was added amine **9** (75 µmol, 0.125 M in DMA, 1.0 equiv), triethylamine (150 µmol, 2 equiv) and HATU (200 µL, 90 µmol, 0.45 M in DMA, 1.2 equiv)**.** The vials were capped and shaken at 50 ^oC. After 24 h, reaction mixtures from selected vials were assessed by LC-MS to ensure complete reaction. The

solvents were evaporated by Genevac and the residue was purified by preparative HPLC to give the final product.

Characterization data for a representative compound **14** prepared by this protocol is listed below. ^{1}H NMR (400 MHz, CD_3OD) δ 6.97 (d, *J* = 8.2 Hz, 2H), 6.85 (d, *J* = 8.2 Hz, 2H), 4.91-4.71 (m, 2H), 3.77-3.63 (m, 2H), 3.41 (q, *J* = 7.2 Hz, 2H), 2.91-2.85 (m, 2H), 2.72 (q, *J* = 7.2 Hz, 2H), 2.60-2.52 (m, 2H), 2.42 (q, *J* = 7.8 Hz, 2H), 1.20 (t, *J* = 7.5 Hz, 3H), 1.37-1.04 (t, *J* = 7.5 Hz, 3H). m/z = 382.2 $(M+H)^+$. HPLC purity 100%, t_R = 0.71 min.

Horner-Wadsworth-Emmons Library Protocol



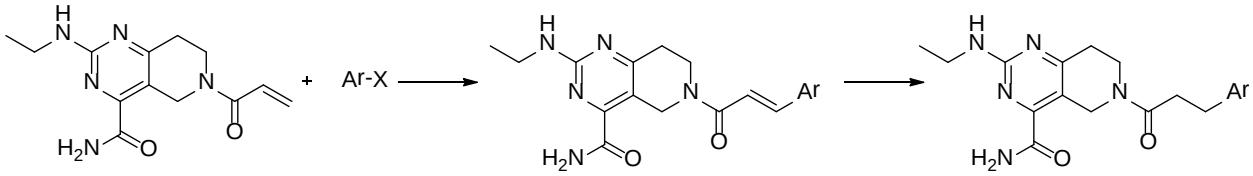
10

To a solution of the phosphonate **10** (1.00 equiv.) in THF (0.05 M) was added LiCl (1.00 equiv) followed by DBU (1.00 equiv). The reaction was stirred for 5 min and then treated with a solution of the appropriate aldehyde (1.00 equiv, 0.1 M in THF) dropwise. The reaction was stirred at room temperature until complete by LCMS analysis (typically 5-15 h). The reaction was poured into saturated sodium bicarbonate and extracted with EtOAc. The organics were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude 3-arylacryl amide, which was used directly in the next step.

The 3-arylacryl amide (1.00 equiv) was dissolved in MeOH (0.02 M) and treated with 10% Pd/C (50% wet for safety, 0.25 equiv) under nitrogen. Triethylsilane (10.0 equiv) was added dropwise, and the reaction mixture was stirred at room temperature until complete by LCMS (typically 1 h). The crude reaction mixture was filtered through Celite and concentrated under reduced pressure. The crude material was purified by reversed phase HPLC to afford the desired product.

Characterization data for a representative compound **17** prepared by this protocol is listed below. ^{1}H NMR (400 MHz, CD_3OD) δ 7.41-7.35 (m, 2H), 7.28-7.23 (m, 2H), 4.95-4.75 (m, 2H), 3.82-3.72 (m, 2H), 3.50-3.38 (m, 2H), 3.03-2.93 (m, 2H), 2.85-2.65 (m, 4H), 1.25-1.15 (m, 3H); m/z = 454.1 $(M+H)^+$. HPLC purity 100%, t_R = 0.73 min

Heck Library Protocol



11

To a solution of the acryl amide **11** (1.00 equiv) in 1,4-dioxane (0.10 M) was added the aryl-halide (1.50 equiv) followed by *N*-cyclohexyl-*N*-methylcyclohexanamine (3 equiv) and Pd(*t*-$Bu_3P)_2$ (0.10 equiv). Nitrogen was bubbled through the reaction mixture for 5 min. The reaction was heated under nitrogen at 100 ^oC until complete by LCMS (typically 5-15 h). The reaction was poured into saturated sodium bicarbonate and extracted with EtOAc. The organics were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude 3-arylacryl amide, which was used directly in the next step.

The 3-arylacryl amide (1 equiv) was dissolved in MeOH (0.02 M) and treated with 10% Pd/C (50% wet for safety, 0.25 equiv) under nitrogen. Triethylsilane (10 equiv) was added dropwise, and the reaction mixture was stirred at room temperature until complete by LCMS (typically 1 h). The crude reaction mixture was filtered through Celite and concentrated under reduced pressure. The crude material was purified by reversed phase HPLC to afford the desired product.

Characterization data for a representative compound **16** prepared by this protocol is provided above.

Supplementary Table 1. MS and HPLC purity for additional analogs

Cmpd	m/z	t_R (min)	HPLC purity (%)[a]
12	354.2	0.47	>99
13	368.2	0.51	>99
5	396.2	0.63	>99
15	384.2	0.45	>99
18	436.2	0.63	>99
19	418.2	0.60	>99
20	419.2	0.52	98
21	423.1	0.72	>99

[a]See General Experimental section for assay conditions. UV detection, 254 nM

Supplementary Table 2. Physicochemical Properties of Comparator Compounds

Cmpd	MW	elogD	TPSA
lithocholic acid	377	2.77[a]	58
1	451	1.85	98
2	375	3.90	46
3	453	5.10	34
4	341	2.90	51

[a] calculated logD

Section 2: Assay Protocols

in vitro cAMP assays

TGR5 is a Gαs-protein coupled that, when stimulated, induces the activation of Adenylate Cyclase (AC), thereby resulting in increases of intracellular cAMP. Agonists of TGR5 were identified using an in vitro HTRF® (Homogeneous Time-Resolved Fluorescence) competitive immunoassay (HTRF® cAMP dynamic 2 Assay Kit; Cis Bio cat # 62AM4PEC) which compares basal cAMP level in whole cells with cAMP levels reached after stimulation with compound. A tracer molecule, d2-labeled cAMP, acts as an acceptor for a Europium (Eu^{3+}) cryptate donor. A monoclonal anti-cAMP antibody has been labeled with Eu-cryptate so that when d2-cAMP binds to the Mab, energy is transferred from the donor to the acceptor.

The complex is excited by light at a wavelength of 340 nm and d2-cAMP binding is detected by emission at wavelength 665 nm. In this assay, intracellular cAMP generated by TGR5 activation competes with the d2-cAMP tracer molecule for binding to the labeled antibody, thus resulting in a change in fluorescence due to the prevention of energy transfer from the donor to the tracer. The fluorescent signal is therefore inversely proportional to the concentration of cellular cAMP resulting from TGR5 activation.

Detecting and calculating the ratio of 665 nm/620 nm emissions allowed sources of interference to be minimized (e.g. medium, colored compounds). The two cell lines used in this assay, Flp-In™-CHO-TO-humanTGR5 and Flp-In™-CHO-TOcanineTGR5, were both constructed using the Flp-In™ T-REx™ System (pcDNA™5/FRT/TO Vector Kit Invitrogen cat# V6520-20). This expression system utilized the tetracycline repressor gene to tightly regulate transcription of the gene of interest (GOI) which, in our case, was human and canine TGR5. In the absence of tetracycline, transcription is blocked and therefore little or no TGR5 is expressed. However, when cells are induced with doxycycline (an analog of tetracycline), transcription of our GOI occurs and very high levels of TGR5 are expressed. The screening cascade we developed utilized both the induced and uninduced cell lines. The induced cell

line, with a high level of receptor expression, provided high sensitivity and a large dynamic range which allowed us to screen compounds with widely varying potencies. The lower-expressing, uninduced cell line provided a more conservative estimate of agonist potency and intrinsic activity.

The growth and assay conditions for both human and canine TGR5 cell lines were identical. To prepare the cells for screening, one vial of cryo-preserved Flp-In™-CHO-TO-humanTGR5 (or Flp-In™-CHO-TO-canineTGR5, respectively) was rapidly thawed in a 37 °C water bath, transferred dropwise into 10 mL of Growth Media (F-12 Nutrient Mixture Ham's (Invitrogen, cat# 11765-054), 10% Tet System Approved Fetal Bovine Serum, US Sourced (Gamma Irradiated) (Clonetech, cat# 631101), 1% Penicillin/Streptomycin (Gibco, cat# 15140), 2 mM L-Glutamine (Gibco cat# 25030), 550 μg/mL Hygromycin B (Invitrogen cat#10687-010), 15 μg/mL Blasticidin S HCl (Invitrogen cat# R210-01) in a 50 mL conical tube, centrifuged at 1000 rpm for 5 min then gently resuspended in 15 mL of fresh Growth Media before added to a T75 flask to incubate at 37 °C, in a 5% CO_2 incubator. When cells reached ~80% confluence, they were expanded into two new flasks, one for induced cells and one for uninduced cells. The media was removed from the T75 flask and cells were washed with room temperature Dulbecco's Phosphate Buffered Saline (PBS) (Sigma cat# D8537). The PBS was aspirated and 5 ml 0.05% Trypsin-EDTA solution (Gibco cat# 25300 was added to the flask and incubated for 2-3 minutes at 37 °C/5% CO_2. The cells were detached by gently tapping the flask, then adding back 10 mL pre-warmed Growth Media to deactivate Trypsin-EDTA. The cell density was adjusted to 1.5 x 10^7 cells/ T175 flask so that 80% confluence could be reached 48 hours after seeding each flask. The cells were allowed to attach for 24 hours, after which one flask was induced with 4 μg/mL doxycycline (prepared in 100% ethanol at 10 mg/mL) before returning the flasks for overnight incubation. On the day of the assay, the cells were harvested and resuspended in Assay Media containing F-12 Nutrient Mixture Ham's (Invitrogen, cat# 11765-054), 0.1% Bovine Serum Albumin Fraction V (heat shock) (Roche Applied Bioscience, cat# 03116999001), Penicillin/Streptomycin (Gibco, cat# 15140), 2 mM L-Glutamine (Gibco cat# 25030), 400 μM isobutylmethylxanthine (IBMX) (Tocris Bioscience, cat#

2845). Cells were re-suspended at a density of 2-4 x 10^5 cells/ml. A total of 5 µl/well of cell suspension was dispensed to all wells of white Greiner 384-well, low-volume assay plate (VWR cat # 82051-458) using a Thermo Multidrop Combi.

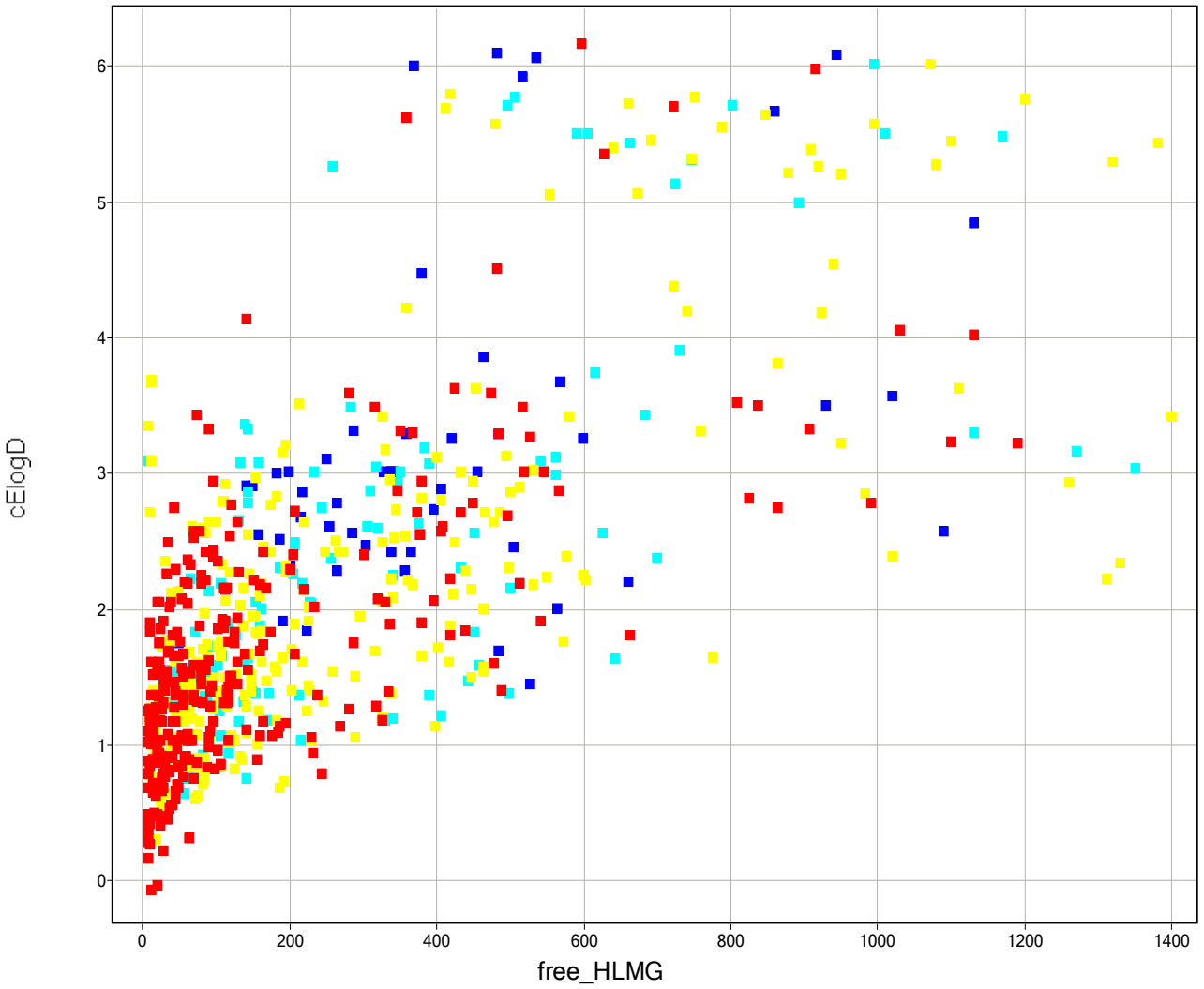
Test compounds were serially diluted in 100% DMSO and spotted 0.5 µL/well to an empty, 384-well, polypropylene plate (Costar # 3654). A reported TGR5 agonist (*S*)-1-(6-fluoro-2-methyl-3,4-dihydroquinolin-1(2H)-yl)-2-(isoquinolin-5-yloxy)ethanone (JP 2006063064) was used as a high control while DMSO was used as the low control. Reference compounds were also used in the assay. All wells of the spotted compound plate were diluted 1:120 with 60 µL/well of assay media. 5 µL was transferred from the compound plate to the assay plate containing 5 µL/well of cells (final compound dilution = 1:240). After 30 minutes incubation at 22 ºC, 5 µL of d2-labeled cAMP and 5 µL of anti-cAMP antibody (both diluted 1:20 in cell lysis buffer as described in the manufacturers assay protocol) were dispensed to each well of the assay plate using a Thermo Multidrop Combi. The plates were incubated at room temperature for 60 minutes and then read with a Perkin-Elmer Envision™ 2104 multilabel plate reader using excitation wavelength of 330 nm and emission wavelengths of 615 nm and 665 nm to detect changes in the HTRF® signal. EC_{50} determinations were made from an agonist response curves analyzed with a curve fitting program using a 4-parameter logistic dose response equation. The intrinsic activity is calculated as the percent of maximal activity of the test compound, relative to the activity of a reported TGR5 agonist (*S*)-1-(6-fluoro-2-methyl-3,4-dihydroquinolin-1(2H)-yl)-2-(isoquinolin-5-yloxy)ethanone (JP 2006063064).

Supplementary Table 3. TGR5 Agonist Potency of Selected Analogs

Compound	*induced* hTGR5 EC_{50}, nM	*n*	*literature* TGR5 EC_{50}, nM	*uninduced* hTGR5 EC_{50}, nM	*n*	*uninduced* dTGR5 EC_{50}, nM	*n*
lithocholic acid	1620±121	2	580[a]	>10000	2	>10000	4
1	166±16	73	820[b]	5540±384	75	4960±1106	30
2	24±9	5	79[c]	316±100	3	445±290	3
3	154±27	18	65[d]	3220±546	12	2060±515	16
4	47±30	4	13[e]	1420±917	3	584±153	4
12	257	1		8320±1019	2	N.T.	--
13	78±19	2		3780±2764	2	5800±2437	3
14	9.2±3.6	11		215±103	6	828±779	4
5	3.6±1.4	11		158±84	7	213±97	4
15	137±81	4		2430±1050	4	4280	1
16	2.3±0.6	23		75±21	45	135±29	37
17	1.6±0.9	8		48±19	13	112±101	5
18	4.7±1.4	7		154±32	12	233±66	8
19	2.7±0.5	8		146±48	6	382±143	10
20	43±6	6		2990±1068	8	3200±653	15
21	2±1	4		83±30	16	220±77	13
22	3.3±1.2	10		112±25	11	303±106	10
23	2.8±1.7	7		43±29	10	407±173	13

EC_{50} values are reported as arithmetic mean ± 95% confidence interval. [a]*J. Med. Chem*. **2008**, *51*, 1831. [b]*J. Med. Chem*. **2009**, *52*, 7958. [c]*J. Med. Chem*. **2009**, 52, 7962. [d]*Bioorg. Med. Chem. Lett*. **2010**, *20*, 5718. [e]*Bioorg. Med. Chem. Lett*. **2010**, *20*, 1363.

Figure S1: Plot of logD vs. free HLM CLint for ~ 750 pyrimidinopiperidine compounds. Higher free intrinsic clearance tracks with higher logD. Hit level induced human TGR5 potency is achieved at logD >2.



Color by potency: 1 2 3 4	Where dark blue (1) EC_{50} 1-10 nM; light blue (2) EC_{50} 11-100 nM; yellow (3) EC_{50} 101-1000 nM; red (4) EC_{50} 1001-9999 nM in the induced human TGR5 assay system

Free HLM = HLM CLint/fu HLM mic

Human Whole Blood Assay

Whole blood from human donors was treated with heparin (1 mL heparin added to 50 mL blood). After indomethacin to a final concentration of 1 μM and compound (final DMSO concentration of 0.1%) were added to 96 well plates, 500 μL of blood per well were aliquotted into 96 well plates and incubated at 37 °C for 30 minutes. LPS to a final concentration of 100 ng/mL was added and then incubated at 37 °C for 4-5 h, mixing every hour to prevent settling of blood. The plates were centrifuged at 2500 rpm for 10 min and 100 μL from each well assayed for TNFα following the manufacturer's instructions for the Millipore Milliplex MAP Human Cytokine/Chemokine Panel kit (catalog number MPXHCYTO-60K). Results were analyzed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

NCI-H716 cAMP assay

NCI-H716 cells were obtained from the ATCC (catalog number CCL-251) and cultured according to their instructions. 50,0000 cells in 45 μL RPMI (Invitrogen catalog number 11875-093) with 0.5 mM IBMX were plated per well in Matrix 96 well plates (Thermo catalog number 4938). Test compounds were serially diluted in RPMI with 0.5 mM IBMX and the cells were treated with compounds for 1 h at 37 ^{o}C. cAMP accumulation was measured and analyzed as described for the recombinantly expressed human and canine TGR5 cAMP assays.

Peripheral Blood Mononuclear Cells (PBMC) cAMP assay

Whole blood from human donors was treated with heparin (1 mL heparin added to 50 mL blood) and mononuclear cells were collected using Sigma-Aldrich Accuspin tubes (catalog number A-7054), following the manufacturer's instructions. Remaining red blood cells were lysed by resuspending the pellet in 9 mL water, then mixed with 1 mL 10X HBSS (Hank's Balanced Salt Solution, Invitrogen catalog number 14065-056). After Phosphate Buffered Saline was added to bring the total volume to 45 mL, tubes were centrifuged at 1200 rpm at room temperature for 10 min. The pellet was resuspended in 5 mL RPMI with 0.1% BSA (bovine serum albumin, Sigma catalog number A7888) then filtered

through a 100 micron cell strainer (BD catalog number 352360). The resulting PBMCs were plated at 50,0000 cells in 45 μL RPMI with 0.1% BSA, 0.5 mM IBMX per well in Matrix 96 well plates. Test compounds were serially diluted in RPMI with 0.5 mM IBMX and the PBMCs were treated with compounds for 1 h at 37 ^{o}C. cAMP accumulation was measured and analyzed as described for the recombinantly expressed human and canine TGR5 cAMP assays.

Section 3: Procedures for in vitro clearance/plasma protein binding/ *in vivo* dog PK

Microsomal Incubations

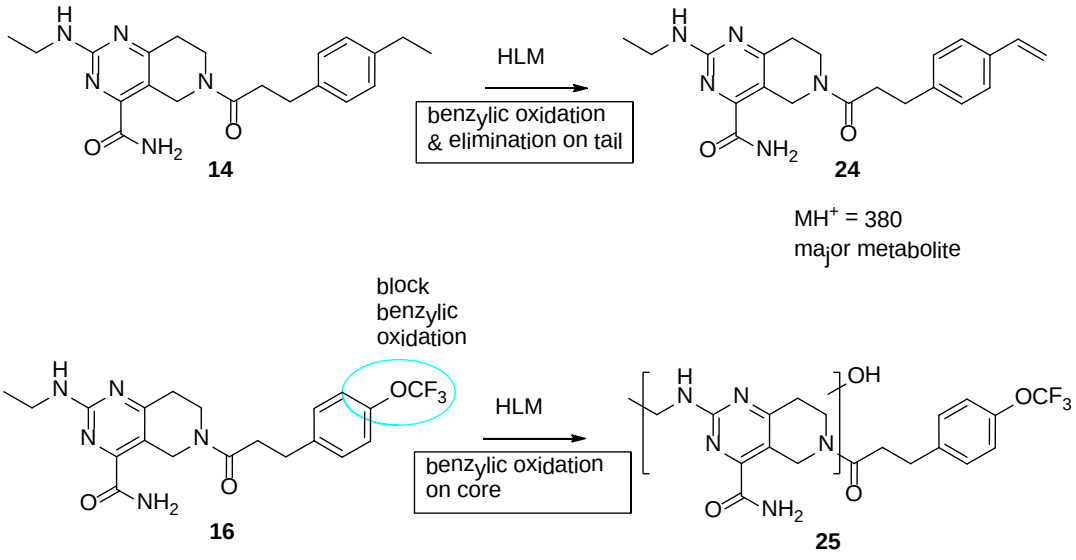
Pooled human liver microsomes and pooled male dog liver microsomes were purchased from BD Biosciences (Woburn, MA). Reaction was conducted in a high throughput screen. Briefly, stock solutions of compound **16** (1 mM in DMSO) were diluted in 50:50 methanol:water (100 μM) and finally to 10 μM working stock in 0.1 M potassium phosphate buffer. The final concentration of DMSO in the incubation media was less than 0.1% (v/v). Microsomal stability assessments were determined after incubation of compound **16** (1 μM) with human or dog liver microsomes (P450 concentration, 0.25 μM) in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mM magnesium chloride, at 37 °C. Incubations were conducted in the presence of NADPH (1 mM). Reaction mixture was quenched with acetonitrile at 0, 5, 10, 20, 30 and 60 minutes post initialization of the reaction. Samples were analyzed via LC-MS/MS for the disappearance of compound **16**. Incubation half-lives and corresponding incubation intrinsic clearance values were determined from analysis of the substrate depletion.[2]

In Vivo Pharmacokinetics

All animal care and in vivo procedures conducted were in accordance with guidelines of the Pfizer Animal Care and Use Committee. Male beagle dogs (~9-12 kg) were used as animal models for pharmacokinetic studies. Dogs were housed one per cage in an American Animal Association Laboratory Animal Care accredited facility with a 12 h light/dark cycle (7:00 AM-7:00 PM). This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US

National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were allowed *ad libitum* access to water and were fasted overnight before dosing and food was returned following the 4 hr blood collection timepoint. Compound **16** was administered intravenously via the cephalic vein (n = 3). For oral studies, **16** was administered by oral gavage to dogs (n = 2). Compound **16** was administered at 1.0 mg/kg i.v. and 3.0 mg/kg p.o. Crystalline compound **16** was formulated as a solution in polyethylene glycol-400, propylene glycol and 30% sulfobutylether-β-cyclodextrin (30:20:50, v/v) and filtered through a 0.22 μm sterile filter for intravenous administration and as a suspension in 0.5% (w/v) methylcellulose for oral studies. After dosing, serial plasma samples were collected at appropriate times and kept frozen at −20°C until LC-MS/MS analysis. Urine samples (0–7.0 and 7.0–24 h) were also collected after intravenous administration.

Figure S2. Metabolite Identification for compounds **14** and **16**



Protein binding

Frozen plasma in K_3EDTA from male beagle dog was purchased from Bioreclamation, Inc. (Westbury, NY). Dulbecco's phosphate buffered saline was purchased from Sigma (Saint Louis, MO). A 96-well equilibrium dialysis apparatus from HTDialysis (Gales Ferry, CT) was used to determine the fu,plasma.[3] Spectra-Por 2 membranes with molecular cutoff of 12 to 14 kDa, obtained from Spectrum

Laboratories Inc. (Rancho Dominguez, CA), were used for the dialysis. The Spectra-Por 2 membranes were conditioned in HPLC grade water for 15 min followed by 30% ethanol for 15 min and 0.10 M sodium phosphate pH 7.4 buffer for 15 min. Frozen plasmas were thawed on the day of the experiment. Untreated plasma and plasma treated with compound **16** (1 μM) and aliquots (150 μL) were loaded into the 96-well equilibrium dialysis plate and dialyzed versus 150 μL of Dulbecco's phosphate-buffered saline buffer. Equilibrium was achieved by incubating the 96-well equilibrium dialysis apparatus on a plate shaker (set at 155 rpm) for 4 hr in a 37 °C CO_2 incubator. After reaching equilibrium, 20 μL of plasma sample and 100 μL of buffer sample aliquots were taken from the 96-well equilibrium dialysis apparatus and added to HPLC vials containing 200 μL of acetonitrile and terfenadine as an internal standard. The appropriate amount of control buffer was added to the plasma and the appropriate amount of control plasma was added to the buffer samples to yield identical matrix between buffer and non-buffer samples. The samples were centrifuged and the supernatant was assayed by LC MS/MS. The percentage of **16** bound to plasma proteins was calculated as: 100 – [(concentration of **16** in buffer/concentration of **16** in plasma) x 100%].[4]

References

(1) (a) Evans, K. A.; Budzik, B. W.; Ross, S. A.; Wisnoski, D. D.; Jin, J.; Rivero, R. A.; Vimal, M.; Szewczyk, G. R.; Jayawickreme, C.; Moncol, D. L.; Rimele, T. J.; Armour, S. L.; Weaver, S. P.; Griffin, R. J.; Tadepalli, S. M.; M. R. Jeune, M. R.; T. W. Shearer, T. W.; Z. B. Chen, Z. B.; Chen, L.; Anderson, D. L.; Becherer, J. D.; De Los Frailes, M.; Colilla, F. J. Discovery of 3-Aryl-4-isoxazolecarboxamides as TGR5 Receptor Agonists. *J. Med. Chem.* **2009**, *52*, 7962-7965. (b) Pinkerton, A. B.; Kabakibi, A.; Herbert, M. R.; Siegel, D. L. PCT Int. Appl. WO2008097976, 2008. (c) Pellicciari, R.; Gioiello, A.; Macchiarulo, A.; Thomas, C.; Rosatelli, E.; Natalini, B.; Sardella, R.; Pruzanski, M.; Roda, A.; Pastorini, E.; Schoonjans, K.; Auwerx, J. Discovery of 6α-Ethyl-23(S)-methylcholic Acid (S-

EMCA, INT-777) as a Potent and Selective Agonist for the TGR5 Receptor, a Novel Target for Diabesity. *J. Med. Chem.* **2009**, *52*, 7958-7961.

(2) Obach, R. S.; Baxter, J. G.; Liston, T. E.; Silber, B. M.; Jones, B. C.; MacIntyre, F.; Rance, D. J.; Wastall, P. The Prediction of Human Pharmacokinetic Parameters from Preclinical and in vitro Metabolism Data. *J. Pharmacol. Exp. Ther*. **1997**, *283*, 46–58.

(3) (a) Pacifici, G. M.; Viani, A. Methods of Determining Plasma and Tissue Binding of Drugs. Pharmacokinetic Consequences. *Clin. Pharmacokinet.* **1992**, *23*, 449-468. (b) Banker, M. J.; Clark, T. H.; Williams, J. A. Development and Validation of a 96-well Equilibrium Dialysis Apparatus for Measuring Plasma Protein Binding. *J. Pharm. Sci*. **2003**, *92*, 967-974.

(4) (a) Kalvass, J. C.; Maurer, T. S. Influence of Nonspecific Brain and Plasma Binding on CNS Exposure: Implications for Rational Drug Discovery. *Biopharm. Drug Dispos*. **2002**, *23*, 327-338. (b) Maurer, T. S.; Debartolo, D. B.; Tess, D. A.; Scott, D. O. Relationship Between Exposure and Nonspecific Binding of Thirty-three Central Nervous System Drugs in Mice. *Drug Metab. Dispos*. **2005**, *33*, 175-181.